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Study on effect of Cissampelos pariera Linn. on glycaemic control and changes in islet morphology of hyperglycamic rats.

Basumata Caroline[1], Sharma Sanilal[2*]
[1]*Associate Professor in Zoology(HOD), Rabindranath Tagore University.*
[2*]*Lecturer in Zoology, Rabindranath Tagore University, Hojai-782435, Assam, India.*
Correspondence: sanilalsharma00@gmail.com.

***Abstract*:**
The present study was aimed to evaluate the anti-diabetic potential of Cissampelos pareira (Menispermaceae) leaf extract (CLE) on fructose-aloxan-induced experimental diabetic rats. Oral administration of water-methanol (1:1) extract of the leaves (100mg and 300mg/kg body weight/day) for 6 weeks and 12 weeks significantly reduced the level of blood glucose, percent glycosylated haemoglobin (%HbA_{1c}) in the diabetic rats in a dose-dependent manner. The standard drug, Pioglitazone (Pioz, 3mg/kg body wt) was used to compare the results. Significant reduction ($P<0.01$) in the % HbA_{1c} levels were observed in the CLE-treated rats. CLE-treated (100mg/Kg bw) animals exhibited significant tolerance for glucose in Oral Glucose tolerance test. Light microscopic studies using Aldehyde-fuchsin staining technique showed significant ($p<0.05$) higher islet volume and β-cells granulation scores in the CLE -treated diabetic rats compared to diabetic control rats.
Key Words: Cissampelos pareira, Menispermaceae, Anti-diabetic, Glycosylated haemoglobin, Glucose.

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I. INTRODUCTION

Diabetes Mellitus is a major and growing health problem in most countries and an important cause of prolonged ill health and early death [1]. According to WHO factsheet 10 November 2021, about 422 million people worldwide have diabetes, the majority living in low-and middle-income countries, and 1.5 million deaths are directly attributed to diabetes each year. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades. Diabetes was predicted to continue to grow worldwide at epidemic proportions in the first quarter of 21st century. The growth was expected to be particularly strong in India and China, which lead the world in the prevalence of diabetes mellitus with 14.3% and 11.8% of prevalence, respectively in 1995 [2, 3]. According to the statistics of diabetes in India 2021, published by Ministry of Health and Family welfare (Published Monday 06 December 2021), the estimated number of diabetes patients in the 20-79 age group is 74.2 millions in 2021 in India and is likely to increase to 124.8 millions in 2045. Nowhere is the diabetes epidemic more pronounced than in India as the World Health Organization (WHO) reports show that 32 million had diabetes in the year 2000 [4]. The National studies on prevalence of diabetes show a rising trend across different parts of India.

Several barriers exist in the treatment of this challenging disease, including the presence of multiple defects and need for a multiple drug treatment approach, the management of postprandial blood glucose, increased rate of hypoglycaemia, treatment related weight gain and metabolic syndrome and drug tolerance. Despite numerous treatments, population studies show no improvement in glycaemic control [5].

There are evidences that diabetes can now be controlled through improved medical care, monitoring, and lifestyle changes. However, the undesirable side effects of certain drugs and poor achievement of glycaemic and other goals have unnerved the patients. Furthermore, the population living below the poverty line is not able to afford the exorbitant cost of drugs and thus rely on herbal medicines. It is not too distant past, a variety of small medical emergencies and ailments were surprisingly effectively controlled and cured by the elders in our families through application of home remedies. They had their own valued recipes passed down from one generation to another, for treating a wide array of health problems. There are several reports on hypoglycaemic activity of some plant [6, 7, 8, 9, 10]. However, few have received medical or scientific scrutiny, and the World Health Organization (WHO) has recommended that traditional treatment for diabetes warrant further evaluation [11]. Systematic survey in remote villages of Karbi Anglong district of Assam in India revealed uses of *Cissampelos* leaves for various ailments by the Bodo tribe in these areas. They also use the juice of the leaves to treat symptoms of diabetes like excessive thirst, polyuria and weakness.

The leaves and roots of *Cissampelos* is reported to have various biological activities [12, 13, 14], but no systematic work on its anti-diabetic activity has been reported in the literature. Hence, the present study was aimed to evaluate the effect of leaf extract of *Cissampelos* on serum glucose, percent glycosylated haemoglobin, and changes in the islet morphology of fructose-aloxan-induced hyperglycaemic and hyperlipidaemic rats. Effect of the extract on triglyceride and cholesterol levels was also evaluated. The effect of *Cissampelos* was compared to pioglitazone, a standard drug which is used to treat Type 2 diabetes in human. Toxicity evaluation of the *Cissampelos* leaf extract was also conducted to avoid any adverse effect prior to conducting above mentioned studies.

II. MATERIALS AND METHODS

1. Chemicals:

Anaesthetic drug (Ketamine hydrochloride) was purchased from Neon Laboratories Ltd.; aloxan was procured from Sigma Chemicals (St. Louis, Mo, USA). The chemical for glucose (Glucose Oxidase-Peroxidase Method), triglyceride (Enzymatic Method), Cholesterol (Enzymatic Method), Serum Glutamate Oxalate Transaminase (SGOT, UV Kinetic IFCC Method), Serum Glutamate Pyruvate Transaminase (SGPT, UV Kinetic IFCC Method) and Alkaline Phosphatase (AP, PNPP Method) were of analytical grade and were purchased from Bayer Diagnostics India Ltd. The chemicals for histological procedures were purchased from Merck Chemicals. The reagent for percent glycosylated haemoglobin determination was purchased from Transasia Bio-medicals Ltd. and ERBA Diagnostics Mannheim/Germany**.**

2. Plant materials:

Cissampelos pareira Linn. (Meispermaceae) plants were collected in the month of June from a remote village in Karbi Anglong district of Assam and planted in the Botanical garden of Hojai College for future use. A voucher specimen of *Cissampelos pareira*was deposited in the herbarium of Botanical Survey of India, Shillong (No.1399/BSI, EC) for further reference.

The leaves of *Cissampelos pareira* Linn. were collected in the months of May, June and July in sunny days, washed properly and air dried. The dried leaves were then powdered in a grinder and stored in a refrigerator at 2-8^{o}C for future use [18].

3. Preparation of the extract and administration:

The leaf powder (10g/100ml) was extracted with water-methanol (1:1) with constant shaking for 24 hours (with short break in between) and filtered. The filtrate was evaporated to dryness in a rotary evaporator below 50^{o}C, weighed and reconstituted with purified water and used (the yield was about 10%).

The animals were first trained to feed from syringe for one to two weeks continuously. When they became accustomed to it, different doses (100mg/kg and 300 mg/kg body weight) of plant extract was continuously fed to the animals between 9 a.m. to 10 a.m. each day during the study period.

4. Anaesthesia:

Rats were anaesthetized with ketamine hydrochloride anaesthesia (Ready-to-use, 100 mg/kg body weight) using 26 gauge needle [19].

5. Animals:

Male Wistar albino rats (*Rattus norvegicus alvinus*) weighing 150-200g (about 6-8 weeks old) were used with the approval of the Institutional Animal Ethical Committee, and were maintained at standard laboratory condition as per the committee's guidelines. Animals were fed standard pellet diet (American Agro.vet. India Ltd.) and water ad-libitum.

6. Experimental animal groups:

The rats exhibiting blood glucose levels in the ranges of 200-250mg/dl and triglyceride levels more than 200mg/dl were randomly selected for the studies and were subdivided into following groups:

Group II: Hyperglycaemic control.

Group III: Hyperglycaemic Pioglitazone (Pioz, 3 mg/kg bw)-treated.

Group IV: Hyperglycaemic CLE-treated. This group was further subdivided into different groups according to different dose.

(Group I: normal control (NC). All the groups consisted of 6 animals).

7. Collection of blood samples from the animals:

The area over the tail, 3cm from the base of the tail, was cleaned with antiseptic scrub. The blood samples were collected by using a syringe equipped with 25 gauge needle inserted at a 45 degree angle towards the vein applying a gentle negative pressure (IACUC Guidelines,Annex VIII). The blood samples were collected in 1.5 ml sterile micro centrifuge tubes and allowed to clot at room temperature. The sera were

separated from the clot within 30 minutes of blood collection and the biochemical parameters were assayed immediately. For insulin determination, 0.1 ml of serum from each animal was aliquoted in the microcentrifuge tubes and kept frozen at -20^{o} C. For Percent Glycosylated Haemoglobin, 0.1 ml of whole blood from each animal was collected in the micro centrifuge tubes containing EDTA.

8. Biochemical parameters:

Prior to conducting the experiments, body weight of the animals was taken and glucose estimations were done in order to rule out congenital glucose intolerance. All the biochemical parameters were performed each week during the study periods.

9. Induction of diabetes in the animals:

They were maintained in a temperature-controlled room at 23°C, with a fixed 12-hour light: 12-hour darkness cycle,and initially fed standard rat laboratory pellet diet (American Agro.vet. India Ltd.) to standardize the nutritional status. Afterone week, the rats were randomly divided into two groups: theexperimental group received 50% Fructose (Fructodex, w/v), and high carbohydrate-source diet, whilethe control rats (NC) received the normal pellet diet.The animals had free access to food and water and were maintainedon their respective diets for 6 weeks. In case of fructose-aloxan-induced diabetic group, the animals were given intra-peritoneal injection of aloxan monohydrate (40mg/kg body weight, w/v) after 6 weeks of fructose feeding. The weight of each animal was recorded twice per week, weight gain of at least 10animals in each group were assessed twice per week during theexperimental period. The food intake of the animals was observed throughout the experimental period. On the day of the experiment, food wasremoved at 09:00 hour the previous night, and experiments were performed between 09:00and 12:00 hour.

10. **Oral Glucose Tolerance Test** (Bajaj S and Srinivasan BP, 1999; Galipeau D, *et al.*, 2001):

Glucose-D (3g/kg bw. weight/volume) was administered to each animal and blood glucose levels were estimated at 0, 30, 60 and 120 minutes. The group IV (1 and 2) of rats was subjected to this test before and after 4 weeks of daily treatment with CLE

STUDIES OF CHANGES IN ISLET MORPHOLOGY OF ANIMALS:

1. Tissue preparation:

Splenic region of pancreas was taken and fixed in Bouin's solution for 24 hours. The tissues were then washed several times with distilled water till the yellow colour of the fixative was completely removed. After dehydrating the tissues in different grades of alcohol (30%, 50%, 70%, 90% and 100%) and clearing with xylene, they were embedded in paraffin blocks and cut in 5 µm thickness and stained with Eosin-Haematoxylene for preliminary observation and finally with Gomori's aldehyde-fuchsin stain. The stained section of highest quality on each slide was chosen for analysis on the basis of lack of artifacts and staining. Each islet was examined by an observer who was unaware of the animal's treatment allocation. Each islet was given a score between 1(least) and 4(greatest) for β-cell granulation observed with aldehyde-fuchsin staining.

2. Scanning of the pancreatic tissues:

Aldehyde fuchsin stained sections from randomly selected pancreases were scanned using micrometer component quantitator [21]. Islet volume, expressed as % of the total splenic region of pancreas was determined at low power (10× objective) with traverse interval of 1mm. The total linear scan for each determination was 500mm or more. The volumes of various components of the islets, expressed as percent of total islet, were measured at higher magnification (40× and oil immersion) with traverse intervals of 20µm.

3. Identification of islet components [20, 21]:

β-cells containing insulin stain purple by Gomori's aldehyde fuchsin stain. In aldehyde fuchsin stained material, β-cells containing varying number of granules, were classified on a scale from 1+ to 4+ granulation. Cells without granules, but with pink cytoplasm were identified as non-granular cells. The vessels identified included the lumen and the endothelial lining of the capillaries within and surrounding the islets.

Because the number of micrometer limited the number of components that could be quantitated per scan to five, the designations were: (1) 3-4+ β-cells (β-cells containing more than half of the full component of granules), (2) 1-2+ β-cells (β-cells containing less than half of the full component of granules), (3) Non-granular cells, and (4) Vessels.

4. Statistical analysis.

All data were presented as mean ±SEM. For data with multiple time points, variables were analyzed by the general linear model ANOVA in the experiment II and by one way ANOVA (Tukey-Kramer multiple

comparison test) by using SPSS version 10.0 in rest of the experiments. An unpaired't' test was also used to compare effect of fructose feeding on the different biochemical parameters. Mean values were considered significant at $p<0.05$, 0.01 and 0.001.

III. RESULTS

1. Oral glucose tolerance test.

The standard drug (Pioz, 3mg/kg b.w.) and CLE (100mg/kg b.w.) treated animals (Group III and IV2 respectively) exhibited significant tolerance for glucose in the oral glucose tolerance test.

The levels of blood glucose in control and experimental group of rats after oral glucose load are shown in Fig. 1. The blood glucose value in the control rats rose to a peak value at 60 min after glucose load and decreased to near normal levels at 120 min. In diabetic control rats, the peak increase in blood glucose concentration was observed after 60 min and remained high over the next 60 minutes. Pioz and CLE (100mg)-treated rats showed significant decrease in blood glucose concentration at 60 and 120 minutes compared to diabetic group of rats ($p<0.001$).

2. Effects of CLE on %HbA_{1c} of high fructose-low aloxan-induced hyperglycaemic rats.

The mean % HbA_{1c} in the normal control group (I) was 6.09 (5.87-6.04) throughout the study period. In the hyperglycaemic control group (II), the mean level was 9.30 (7.89-10.30). Prior to treatment, the mean % HbA_{1c} level in Pioz-treated group (III) was 9.48 (8.60-9.48). After 4 weeks of treatment, the mean level was 8.32 (7.00-9.00), after 8 weeks of treatment, it was 6.74 (6.60- 6.90) and after 12 weeks, the mean level was 6.77 (6.56-7.00).

The mean level in CLE 100mg-treated group (IVB2) was 8.55 (8.41-8.68) before the treatment. The mean levels after 4, 8 and 12 weeks were 7.67 (6.89-7.76), 7.46 (6.89-7.52) and 7.62 (5.89-7.68) respectively (Figure 2).

In the CLE 300 mg treated group (IVC2), the mean % HbA_{1c} levels before the treatment, and at the end of 4, 8 and 12 weeks of treatment period were 9.17 (8.00-9.76), 7.00 (6.49-7.76) , 6.42 (6.40-7.00) and 6.09(5.40-6.88) respectively (Figure 3).

3. Effect of CLE on islet morphology of hyperglycaemic rats:

The Pioz-treated and CLE-treated animals exhibited significantly higher β-cell granulation scores compared to the untreated groups in a dose and time dependent manner.

The islet volumes were significantly reduced in the hyperglycaemic rats. (Plate:2) The average total 3-4+ granulated β-cells were 52.5% in the normal control with 56.9% β-cell volume. (Plate: 1) The average islet volume of the pancreas in the hyperglycaemic animals was 0.35 ±0.04%. The islet volumes were reduced from the control group by 41.2% in the hyperglycaemic rats.

In the Pioz-treated animals, the average scores were 15% (with 28.5% β-cell volume) at the end of 8 weeks. The scores were 28% (with 32% β-cell volume) at the end of 12 weeks of treatment period.In the CLE 100 mg-treated animals, the average total 3-4+ granulated β-cells were significantly higher with 7.8% (with 24% β-cell volume) in the fructose-aloxan group at the end of 8 weeks of continuous treatment. The average score was 8.9% (with 20% β-cell volume) at the end of 12 weeks of treatment (Plate:3).

In the CLE 300mg-treated animals, the average score was 18.5% (with 25% β-cell volume) at the end of 8 weeks and 25% (with 30% β-cell volume) at the end of 12 weeks of continuous treatment (Plate: 4).

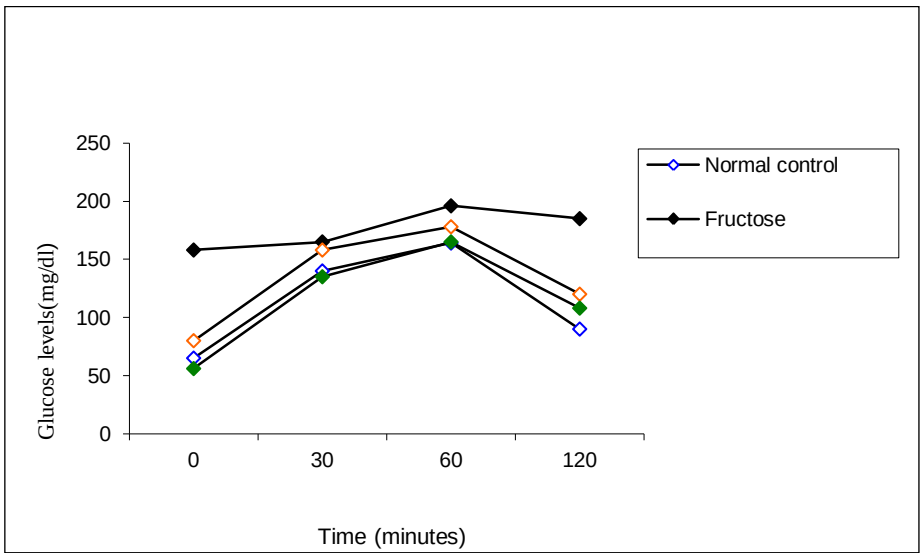

Fig. 1: Oral glucose tolerance test pre- post CLE treatment. The datas are expressed as mean ±SEM (n= 6). The data are significant at p<0.001.

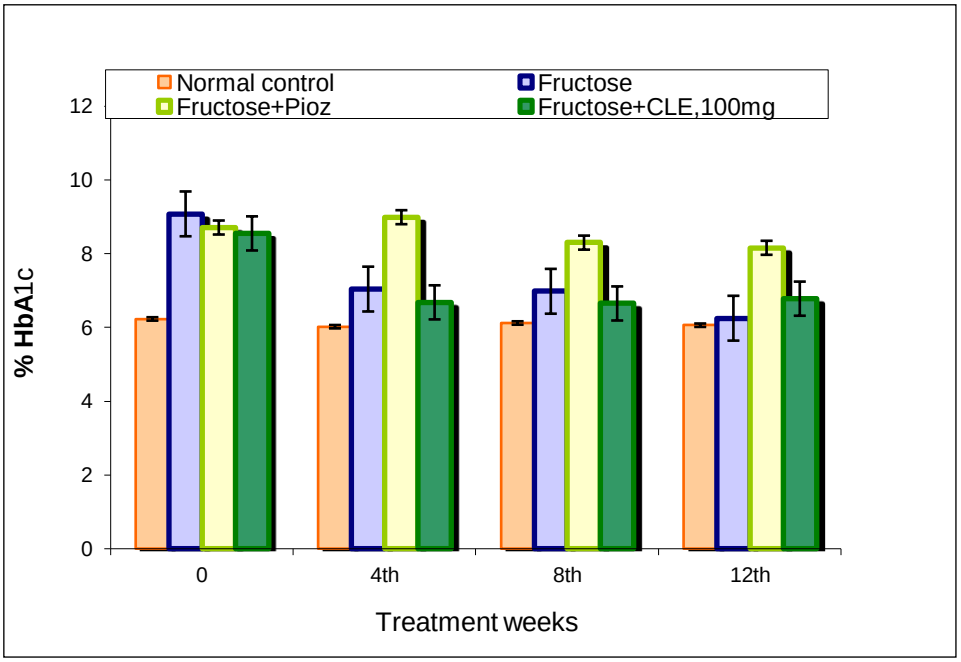

Fig. 2. % HbA_{1c} levels in the standard drug (Pioz, 3mg) and CLE (100 mg) treated **fructose-induced hyperglycaemic rats. Datas are expressed as mean ±SEM (n=6). Datas are significant at P<0.001. Where no error bars are visible the errors were smaller than the symbols.**

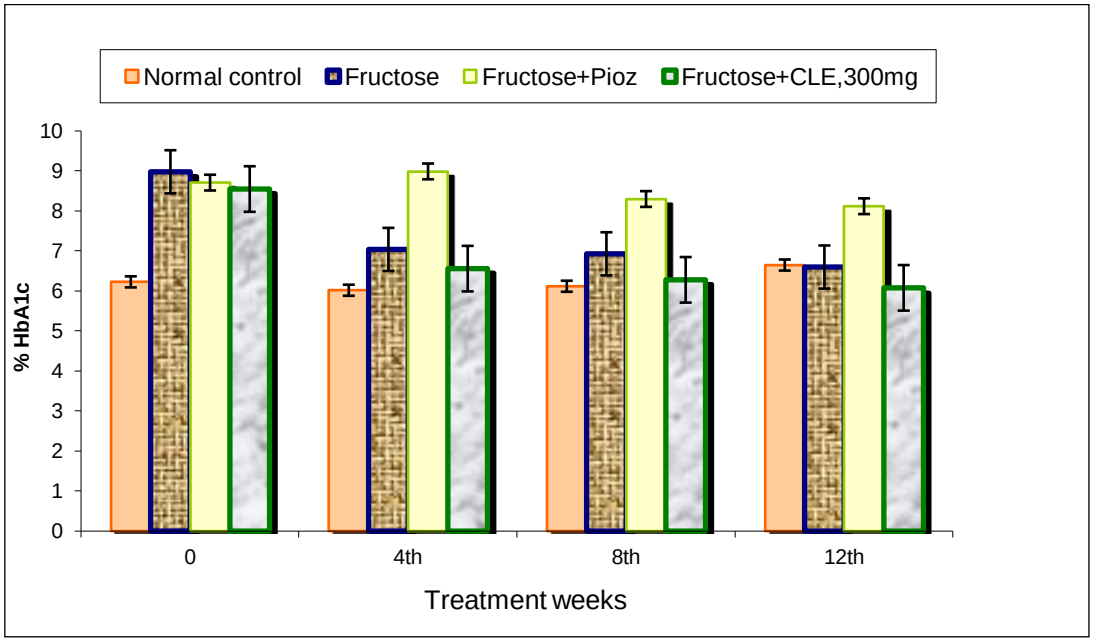

Fig.3. % HbA_{1c} levels in the standard drug (Pioz, 3mg) and CLE (300 mg) treated **fructose-induced hyperglycaemic rats at the end of 12 weeks of treatment period. Datas are expressed as mean ±SEM (n=6). Datas are significant at p < 0. 001.**

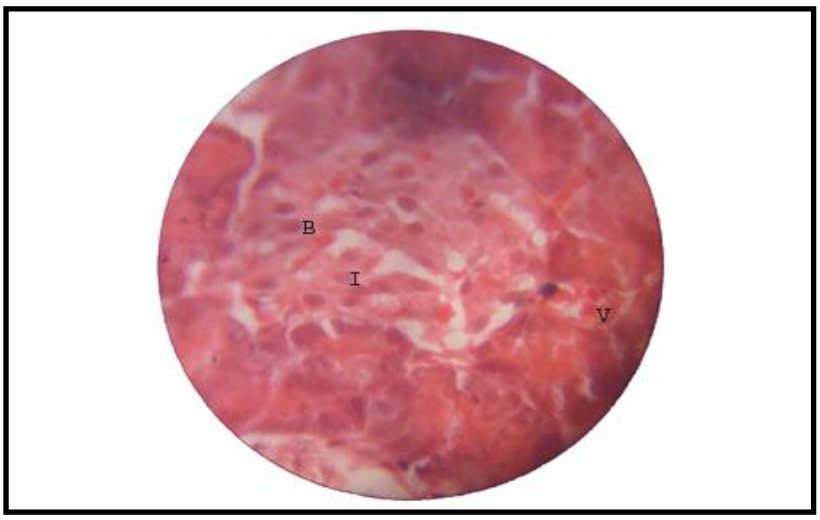

Plate 1. Pancreatic tissue section of normal control rat. ~ × 10. Aldehyde-fuchsin. (I: Islet; B: β-cells; E: Exocrine cells; V: Blood cells).

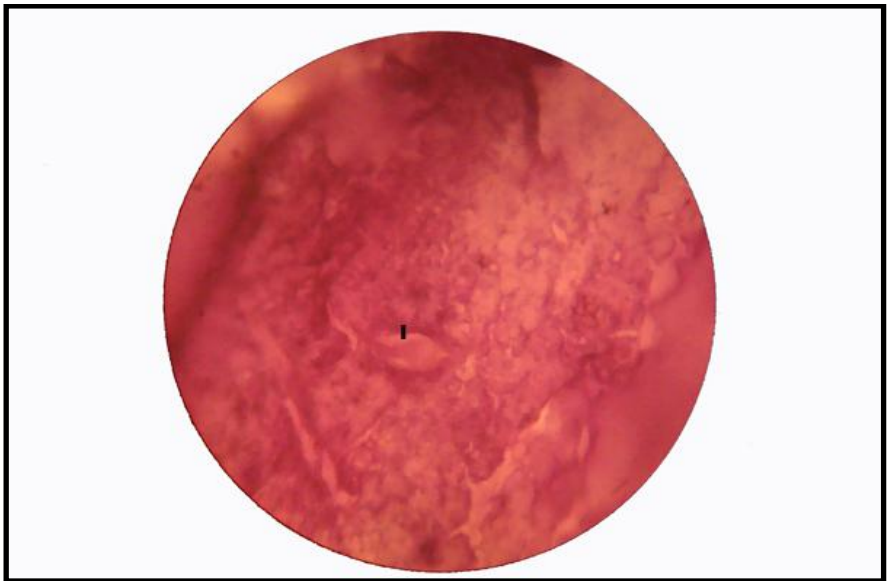

Plate 2. Pancreatic tissue section of fructose-aloxan-hyperglycaemic rat after 7 days of aloxan administration. ~ × 40, Aldehyde-fuchsin. (I: Islet; E: Exocrine cells).

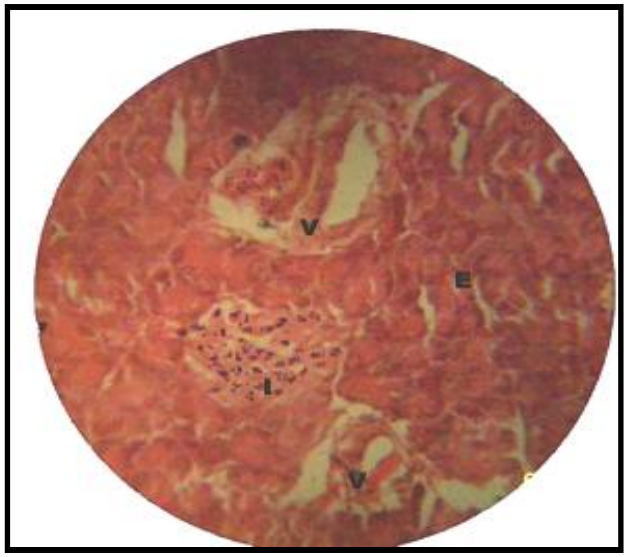

Plate 3. Pancreatic tissue section from Fructose-aloxan + CLE, 100 mg-treated rats after 8 weeks of treatment period. ~ × 40, Aldehyde-fuchsin (I: Islet with β-cells; V: Vessel; E: Exocrine cells).

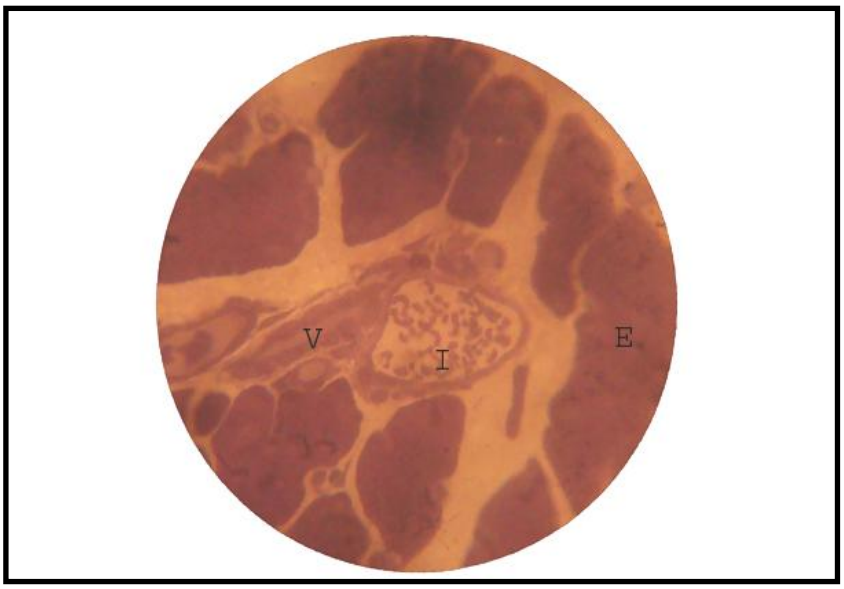

Plate 4. Pancreatic tissue section from Fructose+aloxan+CLE, 300mg- treated rat at the end of 12 weeks of treatment period. ~ × 10, Aldehyde-fuchsin. (I: Islet; V: Vessel; E: Exocrine cells).

IV. DISCUSSION

Aloxan (ALX) is well known for its selective pancreatic islet β-cell cytotoxicity and has been extensively used to induce type 1 and type 2 diabetes in experimental rat models. Due to the reported high incidence of ketosis and resulting mortality, low dose (40 mg/kg body weight) of this agent was used. It was observed that the percentage incidence of diabetic symptoms were quite variable and were not proportionately related to increasing doses of ALX as previously reported by other workers [22] in these ALX treated animals. Therefore, rats were given high fructose [23] and high carbohydrate source diet prior to ALX administration in this experiment. It was interesting to observe that all the animals exhibited significant hyperglycaemia, hyperlipidaemia and other symptoms within 7 days of administration of this drug and persisted till the end of the experimental period.

In this study, the treated animals showed the following signs of the condition: moderate to severe hyperglycaemia, polydipsia (abnormal thirst), polyuria (increased urine volume) weight loss, asthenia (weakness due to the inability to use glucose as source of energy). Histological examination of the pancreatic tissues exhibited reduced % islets and β-cells volume with significantly reduced β-cell granulations that confirms the results found in the study of $\%HbA_{1c}$ and OGTT.

There are evidences that reversal of hyperglycaemia due to pancreatic regeneration is early and common in case of ALX treated animals [24]. However, it has been observed in this study that when high fructose-fed animals were treated with low dose of ALX, the animals exhibited long lasting hyperglycaemia and other symptoms. There was no mortality either in the ALX control group. This may be due to the low dose of aloxan. The long lasting hyperglycaemia and other symptoms exhibited by these animals were due to the administration of high fructose (and also high carbohydrate source diet) to the animals.

Pioglitazone is used as a standard anti-diabetic drug in Type II diabetes [25, 26]. It was used to compare the results in these experiments. The ability of CLE in effectively controlling the increase in blood glucose levels and percent glycosylated haemoglobin in the diabetic rats may be attributed to its anti-hyperglycaemic effects.

There was observed increase in the level of serum insulin in fed rats which indicates that CLE stimulates insulin secretion from the remnants of β-cells or from regenerated β-cells. However, it was observed that there was no significant differences in the levels in the CLE-treated and untreated rats in fasted state. This indicates its different mechanism of action. CLE probably increases insulin action in the tissues.

Increased free fatty acids (FFAs) and triglyceride inhibit insulin-stimulated glucose uptake at the level of glucose transport and/or phosphoryation, inhibit insulin-stimulated glycogen synthesis, and inhibit insulin-stimulated glucose oxidation [27, 28, 29]. FFAs have a special role in the insulin resistance associated with central obesity. Since central adipocytes are more resistant to insulin inhibition of lipolysis, there is an increased delivery of FFAs to the liver. This leads to increased accumulation of triglycerides that could also contribute to

increased hepatic glucose output, reduced hepatic extraction of insulin, and hepatic insulin resistance. The significant reduction in the triglyceride and cholesterol levels of the CLE-treated rats indicates its significant beneficial effect on carbohydrate and fat metabolism.

During diabetes, the excessive glucose in the blood reacts with haemoglobin to form glycosylated haemoglobin [30]. In this study, treatment with CLE showed significant decrease in the percent glycosylated haemoglobin levels in the treated animals. Since decrease in glycohaemoglobin level serves as an indicator of metabolic control in the diabetics [31, 32, 33], the effect of CLE on the $\%HbA_{1c}$ in this diabetic model of rat is noteworthy.

Although it is the normal practice to determine the LD_{50} value, now it is accepted to limit the study with an acute toxicity test using several doses including reasonably high doses of the drugs [18]. In this experiment, acute toxicity of *Cissampelos* was tested up to a high concentration of 10g/kg body weight. The treated animals did not exhibit any toxic symptoms or abnormal behaviour even after administration of such a high dose. There was no mortality either.As it grows annually from perennial rootstock and is found in almost all districts of Assam [34], it can be easily cultivated and further investigation may be carried out to evaluate its beneficial effects on metabolism.

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